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Applicant MEDVET SCIENCE PTY LTD et al	

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I, JANENE PEISKER, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. 2004900266 for a patent by MEDVET SCIENCE PTY. LTD. as filed on 21 January 2004.

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Third day of February 2005

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Regulation 3.

MedVet Science Pty. Ltd.

AUSTRALIA

Patents Act 1990

PROVISIONAL SPECIFICATION

for the invention entitled:

"A Method of Modulating C-Reactive Protein Proinflammatory Activity."

The invention is described in the following statement:

A METHOD OF MODULATING C-REACTIVE PROTEIN PROINFLAMMATORY ACTIVITY.

FIELD OF THE INVENTION

The present invention relates generally to a method of modulating the proinflammatory activity of C-reactive protein in endothelial cells. More particularly, the present invention relates to down-regulation of proinflammatory activity of C-reactive protein in endothelial cells. Accordingly, the method of the present invention is useful, *inter alia*, in the treatment and/or prophylaxis of inflammatory conditions, particularly conditions characterised by proinflammatory activity of C-reactive protein in endothelial cells.

BACKGROUND OF THE INVENTION

Bibliographic details of the publications referred in this specification are referenced at the end of the description. The reference to any prior art document in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that the document forms part of the common general knowledge in Australia.

C-reactive protein (CRP), an acute-phase reactant, has long been considered as a non-specific but sensitive marker of inflammatory diseases including atherosclerosis and associated cardiovascular diseases. More recently, emerging evidence suggests that CRP may have direct proinflammatory effects involved in the pathogenesis of these inflammatory diseases. Elevated CRP has been shown to exert pro-atherogenic effects on vascular cells exemplified by increasing the secretion of monocyte chemoattractant protein (MCP-1),[1] reducing nitric oxide bioactivity,[2,3] and induction of adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and E-selectin.[4] However, constitutive expression of low levels of CRP is detected in normal plasma, and the actual *in vivo* effect of CRP observed from the

experimental animals is controversial.[5] It is possible that the proinflammatory activity of CRP is masked *in vivo* by endogenous neutralizing factors and/or anti-inflammatory agents.

Much recent work reveals that vascular inflammation can be limited by anti-inflammatory counter-regulatory mechanisms that maintain the integrity and homeostasis of vasculature (reviewed in [6]). Previous work by the present inventors shows that high-density lipoproteins (HDL) inhibit the cytokine-induced expression of inflammatory adhesion molecules in endothelial cells (EC), [7] which has been confirmed by many laboratories both *in vitro* and *in vivo*. These findings demonstrate a potent anti-inflammatory capacity of HDL, which could account for the protective effect of HDL against atherogenesis. Indeed, epidemiological and clinical studies showed that HDL concentration is often inversely correlated with the plasma levels of proinflammatory agents, such as cytokines and CRP, in atherosclerotic cardiovascular diseases [8,9] revealing the importance of the balance between anti- and pro-inflammatory potentials in the pathogenesis of these diseases.

In work leading to the present invention, the proinflammatory effect of CRP was measured by the induction of the inflammatory adhesion molecules E-selectin, VCAM-1 and ICAM-1 in human umbilical vein endothelial cells (HUVEC). It has been shown that CRP significantly induced upregulation of adhesion molecules in both protein and mRNA levels. The CRP-induced expression of these inflammatory adhesion molecules was completely suppressed when the cells were preincubated with a physiological concentration (1 mg/ml apoA-I) of high density lipoproteins (HDL) derived from human plasma (native HDL) or reconstituted HDL (rHDL) at a very low concentration (0.01 mg/ml apoA-I). In particular, the CRP-induced upregulation of inflammatory adhesion molecules in HUVEC was completely prevented by HDL via their oxidized phospholipid components. A novel mechanism of HDL inhibition is likely to operate as *a*) rHDL was 100x more potent than native HDL, *b*) pre-incubation with HDL and its sustained presence were obligatory and *c*) oxidized 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (PLPC) was the fundamental active component.

SUMMARY OF THE INVENTION

Throughout this specification and the claims (if any) which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

One aspect of the present invention is directed to a method of modulation of the proinflammatory activity of C-reactive protein in endothelial cells, which comprises administration of an effective amount of an agent which down-regulates, suppresses or inhibits the induction of inflammatory adhesion molecules by C-reactive protein in endothelial cells.

In this aspect, there is more particularly provided a method of down-regulation of the proinflammatory activity of C-reactive protein in endothelial cells, which comprises administration of an effective amount of an agent which down-regulates, suppresses or inhibits the induction of inflammatory adhesion molecules by C-reactive protein in endothelial cells.

In another aspect, the present invention provides a method for the treatment and/or prophylaxis of an inflammatory condition, which comprises administration of an effective amount of an agent which down-regulates, suppresses or inhibits the induction of inflammatory adhesion molecules by C-reactive protein in endothelial cells.

More particularly, in this aspect the present invention provides a method for the treatment and/or prophylaxis of a condition characterised by proinflammatory activity of C-reactive protein in endothelial cells, which comprises administration of an effective amount of an agent which down-regulates, suppresses or inhibits the induction of inflammatory adhesion molecules by C-reactive protein in endothelial cells.

In yet another aspect, the present invention extends to the use of an agent which down-regulates, suppresses or inhibits the induction of inflammatory adhesion molecules by C-reactive protein in endothelial cells in the manufacture of a medicament for the modulation of proinflammatory activity of C-reactive protein in endothelial cells, more particularly for the down-regulation of proinflammatory activity of C-reactive protein in endothelial cells.

In this aspect, the present invention further relates to the use of an agent which down-regulates, suppresses or inhibits the induction of inflammatory adhesion molecules by C-reactive protein in endothelial cells in the manufacture of a medicament for the treatment and/or prophylaxis of an inflammatory condition, more particularly for the treatment and/or prophylaxis of a condition characterised by proinflammatory activity of C-reactive protein in endothelial cells.

In yet another aspect, the present invention provides a pharmaceutical composition for modulation of proinflammatory activity of C-reactive protein in endothelial cells, more particularly for down-regulation of proinflammatory activity of C-reactive protein in endothelial cells, which comprises an agent which down-regulates, suppresses or inhibits the induction of inflammatory adhesion molecules by C-reactive protein in endothelial cells, together with one or more pharmaceutically acceptable carriers and/or diluents.

In this aspect, the present invention further provides a pharmaceutical composition for treatment and/or prophylaxis of an inflammatory condition, more particularly for the treatment and/or prophylaxis of a condition characterised by proinflammatory activity of C-reactive protein in endothelial cells, which comprises an agent which down-regulates, suppresses or inhibits the induction of inflammatory adhesion molecules by C-reactive protein in endothelial cells, together with one or more pharmaceutically acceptable carriers and/or diluents.

In accordance with the present invention, the agent which down-regulates, suppresses or inhibits the induction of inflammatory adhesion molecules by C-reactive protein in endothelial cells may be high density lipoproteins (HDL) derived from human plasma

(native HDL) or reconstituted HDL (rHDL), or a functional derivative thereof. Preferably, the agent is a component of HDL, more preferably a phospholipid component of HDL, and even more preferably an oxidised phospholipid component such as oxidised 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (PLPC). In one embodiment, the agent used to inhibit the induction of inflammatory adhesion molecules by C-reactive protein may be reconstituted high density lipoproteins (rHDL) comprised of apoliprotein AI (Apo-AI) and an oxidised phospholipid component such as oxidised 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (PLPC), or the oxidised phospholipid component alone, for example in the form of small unilamellar vesicles.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows CRP induced adhesion molecule expression. **A.** Flow cytometry profiles show cell-surface expression of E-selectin, ICAM-1 and VCAM-1 in HUVEC treated with CRP (10 μg/ml) for 5 hrs (shaded profiles). The basal levels (solid lines) and negative controls with the isotype-matched, nonrelevant antibodies (dotted lines) are also shown. The mean fluorescence intensity of the positive cells (region M1) is shown in (B) and (C). **B.** Time course of CRP induced adhesion molecule expression. **C.** Dose response of CRP for E-selectin expression in HUVEC stimulated for 5 hrs. $*P<0.05$.

Figure 2 shows CRP-induced adhesion molecule expression is dependent on HUVEC conditioned medium but independent of LPS contamination. **A.** Flow cytometry profiles show cell-surface expression of E-selectin in HUVEC that were untreated (Nil) or treated for 5 hrs with CRP (10 μg/ml) in serum free HUVEC-conditioned Opti-MEM medium (-FCS) or M199 medium containing 20% FCS (+FCS). **B.** Conditioned medium was replaced by fresh medium containing the indicated concentrations of conditioned medium immediately prior to CRP stimulation. E-selectin expression by HUVEC was then assayed. **C.** E-selectin was measured in HUVEC stimulated for 5 hrs with the indicated concentrations of untreated CRP or LPS, or with CRP or LPS that had been heated at 65°C for the indicated periods of time in serum free medium. $*P<0.05$; $**P<0.01$.

Figure 3 shows HDL inhibits CRP-induced adhesion molecule expression. HUVEC were stimulated with CRP (10 μg/ml) for 5 hrs after preincubation for 16 hrs with varying concentrations of native HDL (A) or rHDL (C), then the expression of adhesion molecules was assayed as indicated. **P*<0.05 ***P*<0.01. **B.** The mRNA levels of E-selectin, VCAM-1, ICAM-1 and GAPDH (as a control) were assayed by RT-PCR in HUVEC preincubated for 16 hrs in serum free medium with native HDL (1mg/ml) or rHDL (10 μg /ml) followed by CRP stimulation for the indicated time.

Figure 4 shows inhibitory activity of HDL on CRP is reproduced by PLPC alone. **A.** HUVEC were incubated for 16 hrs in serum free medium with the indicated concentrations of native HDL, rHDL, PLPC or Apo-A1 and stimulated for a further 5 hrs with CRP (10 μg/ml), then the expression of adhesion molecules was assayed as indicated. **B.** Representative histogram of CRP-induced E-selectin expression (stimulated as in A) following 16 hrs preincubation in the presence or absence of PLPC (35 μM).

Figure 5 shows HDL inhibits adherence of U937 cells to BAEC. BAECs were treated with TNF (0.5 ng/ml) or CRP (10 μg/ml) for 24 hrs in the presence or absence of rHDL (10 μg/ml). The pre-labelled U937 cells were incubated with the treated-BAECs for 30 min. **A.** Adherence of U937 cells was microscopically photographed (20X), and **(B.)** determined by visually counting 4 microscopic fields per well in triplicate. **P*<0.001.

Figure 6 shows PLPC activity is dependent on HUVEC conditioned medium. **A.** Unconditioned rHDL or PLPC SUVs were preincubated with HUVEC for 16 hrs or 1 hr with or without washout (W/O), or the HUVEC-conditioned (HC) rHDL or PLPC SUVs (detailed in 'Methods') were preincubated with HUVEC for 1 hr, the cells were then stimulated with CRP for 5 hrs and E-selectin expression was assayed. **P*<0.05. **B.** Mass spectrometric analysis of PLPC prior to incubation with HUVEC (main panel). The inner panel shows the analysis of the lipid extracted from the serum-free medium following overnight incubation of PLPC with HUVEC.

Figure 7 shows oxidation of PLPC eliminates the CRP induced response. The expression of E-selectin was assayed in (**A**) HUVEC preincubated for 16 hrs with 35 μM PLPC in the presence of varying concentrations of α-tocopherol, or (**B**) HUVEC stimulated for 5hrs with either CRP (10 μg/ml) or TNF (0.1 ng/ml) added simultaneously with 35 μM non-oxidized (non-ox) or oxidized PLPC (ox). $^{*}P<0.01$.

DETAILED DESCRIPTION OF THE INVENTION

C-reactive protein (CRP), a well-recognised marker of atherosclerosis, has been recently suggested to have a direct proinflammatory effect. The constitutive expression of low levels of CRP in normal plasma indicates a natural factor may exist to neutralise CRP's effect. The present inventors have shown that the CRP-induced expression of the inflammatory adhesion molecules E-selectin, VCAM-1 and ICAM-1 in human umbilical vein endothelial cells (HUVEC) was completely suppressed when the cells were preincubated with high density lipoproteins (HDL). Furthermore, it has been found that the oxidized form of the phospholipid component of rHDL, was a central molecule responsible for the inhibitory effect of HDL on CRP.

Accordingly, in one aspect the present invention provides a method of modulation of the proinflammatory activity of C-reactive protein in endothelial cells, which comprises administration of an effective amount of an agent which down-regulates, suppresses or inhibits the induction of inflammatory adhesion molecules by C-reactive protein in endothelial cells.

More particularly, in this aspect, the present invention provides a method of down-regulation of the proinflammatory activity of C-reactive protein in endothelial cells which comprises administration of an effective amount of an agent which down-regulates, suppresses or inhibits the induction of inflammatory adhesion molecules by C-reactive protein in endothelial cells.

Reference to "modulation" or "modulating" throughout this specification should be understood as including reference to down-regulation or down-regulating the subject proinflammatory activity. Reference to "down-regulation" or "down-regulating" this activity should be understood as a reference to preventing or suppressing, reducing (e.g. slowing), or otherwise inhibiting one or more aspects of the proinflammatory activity (for example, retarding or preventing the proinflammatory activity).

The agent which down-regulates, suppresses or inhibits the induction of inflammatory adhesion molecules by C-reactive protein in endothelial cells may be high density lipoproteins (HDL) derived from human plasma (native HDL) or, preferably, reconstituted HDL (rHDL), or a functional derivative thereof.

Reference to "functional derivative" of HDL in this specification should be understood to include fragments, parts, portions or variants from either natural or non-natural sources. References to "functional derivatives" of HDL should be understood as references to such derivatives which retain at least one of the functions of HDL, in particular its activity in down-regulating, suppressing or inhibiting the induction of inflammatory adhesion molecules by C-reactive protein in endothelial cells. Functional parts or fragments include, for example, active regions of the HDL molecule. Non-natural sources of HDL or its derivatives include, for example, recombinant or synthetic sources. By "recombinant sources" it is meant that the cellular source from which the subject molecule is harvested has been genetically altered. This may occur, for example, in order to increase or otherwise enhance the rate and volume of production by that particular cellular source.

As previously described, the agent used to inhibit the induction of inflammatory adhesion molecules by C-reactive protein may be reconstituted high density lipoproteins (rHDL) comprised of apoliprotein AI (Apo-AI) and an oxidised phospholipid component such as oxidised 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (PLPC), or the oxidised phospholipid component alone, for example in the form of small unilamellar vesicles.

Derivatives may also be derived from insertion, deletion or substitution of amino acids. Amino acid insertional derivatives include amino and/or carboxylic terminal fusions as well as intrasequence insertions of single or multiple amino acids. Insertional amino acid sequence variants are those in which one or more amino acid residues are introduced into a predetermined site in the protein, although random insertion is also possible with suitable screening of the resulting product. Deletional variants are characterised by the removal of one or more amino acids from the sequence. Substitutional amino acid variants are those in which at least one residue and its sequence has been removed and a different residue inserted in its place. Additions to amino acid sequences include fusion with other peptides, polypeptides or proteins.

In one particularly preferred embodiment of the present invention, the agent which down-regulates, suppresses or inhibits the induction of inflammatory adhesion molecules by C-reactive proteins in endothelial cells is reconstituted HDL (rHDL), for example rHDL comprising a recombinant mutant form of Apo-AI complexed with phospholipid[28].

In another particularly preferred embodiment of the present invention, this agent which down-regulates, suppresses or inhibits the induction of inflammatory adhesion molecules by C-reactive protein in endothelial cells is a component of HDL, more preferably a phospholipid component of HDL, and even more preferably an oxidised phospholipid component such as oxidised PLPC. Thus, in the work leading to the present invention, CRP-induced upregulation of inflammatory adhesion molecules in HUVEC was completely prevented by HDL via the oxidised phospholipid components thereof.

In another aspect, the present invention provides a method for the treatment and/or prophylaxis of an inflammatory condition which comprises administration of an effective amount of an agent which down-regulates, suppresses or inhibits the induction of inflammatory adhesion molecules by C-reactive protein in endothelial cells.

More particularly, in this aspect the present invention provides a method for the treatment and/or prophylaxis of a condition characterised by proinflammatory activity of C-reactive

protein in endothelial cells, which comprises administration of an effective amount of an agent which down-regulates, suppresses or inhibits the induction of inflammatory adhesion molecules by C-reactive protein in endothelial cells.

An "effective amount" means an amount necessary at least partly to attain the desired response, or to delay the onset or inhibit progression or halt altogether, the onset or progression of the particular condition being treated. The amount varies depending upon the health and physical condition of the individual to be treated, the taxonomic group of the individual to be treated, the degree of protection desired, the formulation of the composition, the assessment of the medical situation, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials.

Reference herein to "treatment" and "prophylaxis" is to be considered in its broadest context. The term "treatment" does not necessarily imply that a subject is treated until total recovery. Similarly, "prophylaxis" does not necessarily mean that the subject will not eventually contract a disease condition. Accordingly, treatment and prophylaxis include amelioration of the symptoms of a particular condition or preventing or otherwise reducing the risk of developing a particular condition. The term "prophylaxis" may be considered as reducing the severity or onset of a particular condition. "Treatment" may also reduce the severity of an existing condition.

The present invention further contemplates a combination of therapies, such as the administration of the modulatory agent together with other proteinaceous or non-proteinaceous molecules which may facilitate the desired therapeutic or prophylactic outcome.

Administration of an active agent in accordance with the present invention as hereinbefore described [herein collectively referred to as "modulatory agent"], in the form of a pharmaceutical composition, may be performed by any convenient means. The modulatory agent of the pharmaceutical composition is contemplated to exhibit therapeutic

activity when administered in an amount which depends on the particular case. The variation depends, for example, on the human or animal and the modulatory agent chosen. A broad range of doses may be applicable. Considering a patient, for example, from about 0.1 mg to about 1 mg of modulatory agent may be administered per kilogram of body weight per day. Dosage regimes may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily, weekly, monthly or other suitable time intervals or the dose may be proportionally reduced as indicated by the exigencies of the situation.

The modulatory agent may be administered in a convenient manner such as by the oral, intravenous (where water soluble), intraperitoneal, intramuscular, subcutaneous, intradermal or suppository routes or implanting (e.g. using slow release molecules). The modulatory agent may be administered in the form of pharmaceutically acceptable nontoxic salts, such as acid addition salts or metal complexes, e.g. with zinc, iron or the like (which are considered as salts for purposes of this application). Illustrative of such acid addition salts are hydrochloride, hydrobromide, sulphate, phosphate, maleate, acetate, citrate, benzoate, succinate, malate, ascorbate, tartrate and the like. If the active ingredient is to be administered in tablet form, the tablet may contain a binder such as tragacanth, corn starch or gelatin; a disintegrating agent, such as alginic acid; and a lubricant, such as magnesium stearate.

Routes of administration include, but are not limited to, respiratorally, intratracheally, nasopharyngeally, intravenously, intraperitoneally, subcutaneously, intracranially, intradermally, intramuscularly, intraoccularly, intrathecally, intracereberally, intranasally, infusion, orally, rectally, *via* IV drip, patch and implant.

In accordance with these methods, the modulatory agent in accordance with the present invention may be coadministered with one or more other compounds or molecules. By "coadministered" is meant simultaneous administration in the same formulation or in two different formulations via the same or different routes or sequential administration by the same or different routes. By "sequential" administration is meant a time difference of from

seconds, minutes, hours or days between the administration of the two types of molecules. These molecules may be administered in any order.

Another aspect of the present invention relates to the use of an agent which down-regulates, suppresses or inhibits the induction of inflammatory adhesion molecules by C-reactive protein in endothelial cells in the manufacture of a medicament for the modulation of proinflammatory activity of C-reactive protein in endothelial cells, more particularly for the down-regulation of proinflammatory activity of C-reactive protein in endothelial cells.

In another aspect the present invention relates to the use of an agent which down-regulates, suppresses or inhibits the induction of inflammatory adhesion molecules by C-reactive protein in endothelial cells in the manufacture of a medicament for treatment and/or prophylaxis of an inflammatory condition, more particularly in the treatment and/or prophylaxis of a condition characterised by proinflammatory activity of C-reactive protein in endothelial cells.

In yet another aspect, the present invention provides a pharmaceutical composition for modulation of proinflammatory activity of C-reactive protein in endothelial cells, more particularly for down-regulation of proinflammatory activity of C-reactive protein in endothelial cells, which comprises an agent which down-regulates, suppresses or inhibits the induction of inflammatory adhesion molecules by C-reactive protein in endothelial cells, together with one or more pharmaceutically acceptable carriers and/or diluents.

In yet another further aspect, the present invention contemplates a pharmaceutical composition for treatment and/or prophylaxis of an inflammatory condition, more particularly treatment and/or prophylaxis of a condition characterised by proinflammatory activity C-reactive protein in endothelial cells, which comprises an agent which down-regulates, suppresses or inhibits the induction of inflammatory adhesion molecules by C-reactive protein in endothelial cells, together with one or more pharmaceutically acceptable carriers and/or diluents.

The methods, uses and compositions as broadly described herein are particularly applicable in the treatment and/or prophylaxis of mammalian subjects.

The term "mammal" and "subject" as used herein includes humans, primates, livestock animals (eg. sheep, pigs, cattle, horses, donkeys), laboratory test animals (eg. mice, rabbits, rats, guinea pigs), companion animals (eg. dogs, cats) and captive wild animals (eg. foxes, kangaroos, deer). Preferably, the mammal is human or a laboratory test animal Even more preferably, the mammal is a human.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion or may be in the form of a cream or other form suitable for topical application. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of superfactants. The preventions of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilisation. Generally, dispersions are prepared by incorporating the various sterilised active ingredient into a sterile vehicle

which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze-drying technique which yield a powder of the active ingredient plus any additional desired ingredient from previously sterile-filtered solution thereof.

When the active ingredients are suitably protected they may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or it may be enclosed in hard or soft shell gelatin capsule, or it may be compressed into tablets, or it may be incorporated directly with the food of the diet. For oral therapeutic administration, the active compound may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 1% by weight of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 5 to about 80% of the weight of the unit. The amount of active compound in such therapeutically useful compositions in such that a suitable dosage will be obtained. Preferred compositions or preparations according to the present invention are prepared so that an oral dosage unit form contains between about 0.1 µg and 2000 mg of active compound.

The tablets, troches, pills, capsules and the like may also contain the components as listed hereafter: a binder such as gum, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, lactose or saccharin may be added or a flavouring agent such as peppermint, oil of wintergreen, or cherry flavouring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compound, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavouring such as cherry or orange flavour. Of

course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compound(s) may be incorporated into sustained-release preparations and formulations.

The present invention is further defined by the following non-limiting Example.

EXAMPLE

Material and Methods

Cell Culture and Flow Cytometry Analysis. Human umbilical vein endothelial cells (HUVEC) and bovine aortic endothelial cells (BAEC) were isolated and cultured as described previously [10]. Cells were used at passages 2 to 3 for HUVEC and passages 3-10 for BAEC. For detection of adhesion molecules, HUVEC were incubated overnight in Opti-MEM serum free medium (Gibco, Invitrogen Corporation) in the presence or absence of HDL and then treated with recombinant human CRP (Calbiochem) for 5 hrs unless indicated otherwise. After the treatment, cells were washed with medium M199 and incubated with primary monoclonal antibodies against E-selectin, VCAM-1, ICAM-1 or isotype-matched nonrelevant control antibodies for 30 min as described previously[11]. Cells were then incubated with fluorescein isothiocyanate-conjugated secondary antibody for 30 min. The cells were then harvested by trypsinization and fixed in 2.5% formaldehyde (antibody binding prior to trypsinizing has been reported to prevent the partial hydrolyzation of the surface adhesion molecules [12]). The expression of cell-surface adhesion molecules was measured as fluorescence intensity by use of a Coulter Epics Profile XL flow cytometer. Unless stated otherwise, the results represent mean fluorescence of the positive population ± SEM from one experiment and are representative at least three independent experiments. Differences between means were evaluated by Student's *t*-tests. ANOVA was used to identify statistical significance of multiple comparisons.

Adherence of U937 cell to EC. U937 cells (CRL 1593.2; ATCC) were colorimetrically labelled with 0.2 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Promega) in normal culture medium for 30 min at 37°C. The cells were collected by low-speed centrifugation and resuspended at a density of 2×10^5 cells/ml in medium without FCS. BAECs were seeded into 24-well plates and treated as desired in triplicate. After the treatment, BAECs were washed twice with RPMI-1640 medium. The MTT-labelled U937 cell suspension (200 µl/well) was then added into the BAEC cultures and incubated for 30 min at 37°C. Non-adherent cells were removed by rinsing the plates three times with PBS, and the number of adherent cells was counted under microscopy at least 4 fields per well.

Isolation and Preparation of Lipoproteins and small unilamellar vesicles. As described previously[13] the lipoproteins were isolated from normal healthy adult donors by sequential ultracentrifugation in their appropriate density range: total HDL $1.07 < d < 1.21$ and LDL $1.019 < d < 1.055$ g/ml. The resulting preparations of lipoproteins were dialyzed against endotoxin-free PBS (pH 7.4) prior to use. Discoidal reconstituted HDL containing apoA-I and PLPC, were prepared by the cholate dialysis method described by Matz *[14]*. Small unilamellar vesicles (SUVs) containing either PLPC or 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), in butylated hydroxytoluene (BHT) (molar ratio of PC to BHT, 10:1), were prepared in PBS precisely as described by Jonas [15]. Oxidation of SUV was produced by incubation with 5 µM $CuSO_4$ in the absence of BHT for 48 hours at 37°C.

Mass Spectrometry. Samples of phospholipid were extracted with $CHCl_3$:CH_3OH (2:1), diluted 10-fold in acetonitrile + 0.05% formic acid. Samples (20 µl) were infused at 10 µl/min into a PE/Sciex API-100 electrospray-ionization mass spectrometer (PE Biosystems, Melbourne, Australia) with ionization at 4,000V and the orifice set at 65V and the spectra acquired (range 200-1000Th at 0.1Th resolution).

RT-PCR. The primers used to amplify E-selectin, VCAM-1 and ICAM-1 were as described in [16] and were designed to span intron-exon boundaries. Total RNA was extracted from

HUVEC using TRIzol (Gibco BRL) according to the manufacturers instructions. First-strand cDNA was synthesised from 1 μg total RNA using Omniscript reverse transcriptase (QIAGEN) and ADAPTOR primer (Geneworks). E-selectin, VCAM-1 and ICAM-1 were amplified over 27 cycles with an internal GAPDH control. Amplified products were visualised by electrophoresis on 1.5% agarose gel stained with ethidium bromide.

Results

CRP stimulates adhesion molecule expression in conditioned serum-free medium.

As the induction of adhesion molecules by EC is critical for proinflammatory reactions in the vasculature, we examined the effect of CRP on expression of VCAM-1, ICAM-1 and E-selectin in HUVEC. Treatment of HUVEC with CRP resulted in a significant increase in the cell surface expression of E-selectin VCAM-1 and ICAM-1 (Figure 1A). The time-course of CRP induced adhesion protein expression was similar to the effect of cytokines such as TNF (Figure 1B). The activity of CRP was dose-dependent, reaching a maximum at 10-20 μg/ml (Figure 1C) ranged in pathophysiological concentrations that are often seen in chronic inflammatory diseases including atherosclerosis.

Pasceri et al [4] previously reported that the CRP-induced expression of adhesion molecules was serum-dependent. We found that CRP was capable of inducing adhesion molecule expression in HUVEC cultured in either medium containing 20% FCS or serum-free Opti-MEM medium that had been conditioned by HUVEC for 16 hours (Figure 2A). However, there was no inflammatory effect when CRP was added to cells in fresh serum-free medium (Figure 2B), which was consistent with Pasceri's report [4]. CRP induced adhesion molecule expression was restored when HUVEC conditioned medium was added back to the cells in a concentration-dependent manner (Figure 2B). These data indicate that the effect of CRP is dependent on a factor that is secreted by EC or present in serum.

Lipopolysaccharide (LPS) is known to induce adhesion molecule expression, therefore it was necessary to exclude LPS contamination as a factor in the CRP induced effect. LPS at concentrations of up to 1 ng/ml was insufficient to induce adhesion molecule expression (Figure 2C), while the contamination of LPS detected in the purified CRP was

below 0.1ng/ml. Additionally, heating of CRP at 65°C significantly decreased the inflammatory effect in a time-dependent manner and heating for 1 hour completely eliminated the CRP's activity, whereas heating at 65°C for 1 hour did not alter LPS's effect (Figure 2C). These data strongly indicate that LPS is not responsible for the observed inflammatory effect of CRP.

HDL inhibits CRP-induced expression of adhesion molecules.

Remarkably, the CRP-induced expression of adhesion molecules was profoundly inhibited by native HDL in serum-free medium in a concentration-dependent manner (Figure 3A). Native HDL at a physiological level (1mg/ml of apoA-I) completely blocked the effect of CRP on expression of E-selectin, ICAM-1 and VCAM-1. Additionally, the mRNA levels of these adhesion molecules induced by CRP were also significantly reduced by HDL (Figure 3B). In order to minimise possible confounding effects of the heterogeneity of native HDL particles[13] and of any co-isolated contaminants, we investigated the effects of reconstituted HDL (rHDL). Pre-incubation of HUVEC with rHDL containing PLPC and apoA-I (molar ratio 100:1) resulted in a marked reduction in the CRP-induced expression of E-selectin, VCAM-1 and ICAM-1 (Figure 3C). Complete inhibition of the expression by rHDL was attained at a 100-fold lower concentration of HDL particles (10 μg/ml apoA-I) in comparison to native HDL (Figure 4A). However, treatment with lipid-free apoA-I had no effect (Figure 4A). In contrast, PLPC presented to the cells as SUVs had a similar inhibitory effect to rHDL (Figure 4A and 4B), suggesting a major role of the unsaturated phospholipids in the inhibitory activity of HDL. As controls, pre-incubation of HUVEC with LDL or POPC SUVs had no inhibitory effect on CRP's activity (data not shown).

HDL inhibits adherence of U937 cells to BAEC.

To verify the biological consequences of adhesion molecule expression influenced by CRP and HDL, leukocyte adherence to aortic EC was determined. Figure 5 shows that adhesion of U937 cells increased more than 6-fold following incubation of BAEC with CRP for 24 hours, comparable to the level of binding following TNF stimulation. The induction of adhesion molecules measured by their mRNA levels was similar to that in

HUVEC (data not shown). Significantly, in the presence of rHDL, the number of U937 cells binding to the CRP- or TNF-activated BAEC was markedly reduced (Figure 5).

The mechanism of HDL inhibition on CRP differs from that on cytokine-induced adhesion molecule expression.

The ability of HDL to inhibit cytokine- (such as TNFα or IL-1) induced adhesion protein expression has been well documented [17], we thus investigated whether the inhibitory effect of HDL on CRP is mediated by a common mechanism of inhibition on the adhesion molecule expression induced by cytokines. In previous reports, we showed that a short term pre-incubation (less than 1 hour) with HDL was sufficient for reduction in the TNF-induced expression of VCAM-1 [17], and that the inhibition did not require HDL to be physically present during the activation of adhesion molecule expression by TNF [17,18]. However, no inhibitory effect on CRP-induced adhesion molecule expression was discernible following a 1-hour pre-incubation with either rHDL or PLPC (Fig. 6A), or when these reagents were added simultaneously with CRP (data not shown). Furthermore, when HDL or PLPC were removed from the medium following a 16-hour pre-incubation before activation of the cells with CRP, the inhibitory effect did not persist (Fig 6A). Additionally, in contrast to our previous findings of the inability of phospholipids alone to suppress the TNF-induced adhesion protein expression [7,19], PLPC had a similar inhibitory effect to whole HDL particles on CRP (Fig 4, 6A). Thus, these data suggest different mechanisms underlying HDL inhibition of the CRP- and cytokine-induced proinflammatory actions.

Oxidation of PLPC is required to inhibit the CRP proinflammatory effect.

The inhibitory activity of rHDL or PLPC depends on a prolonged pre-incubation or pre-conditioning by cultured HUVEC (Figure 6A), suggesting that an interaction with EC is required for the rHDL or PLPC inhibitory effect on CRP. CRP has recently been shown to bind to the phosphorylcholine (PC) head group of oxidized LDL and oxidized phospholipids, albeit the biological consequences of the binding are yet unknown [20]. We therefore investigated whether oxidation of PLPC is involved in the PLPC-dependent

inhibition of CRP proinflammatory effect. Mass spectrometric analysis of unoxidized PLPC revealed a single predominant ion peak at m/z 758.7 (Figure 6B), which was lost following incubation of PLPC with cultured HUVEC for 16 hrs, presumably due to oxidation (Figure 6A, inner panel). Interestingly, the PLPC-dependent inhibition of CRP-induced E-selectin expression was reversed in a dose-dependent manner by the presence of the antioxidants α-tocopherol (Figure 7A) or nordihydroguaiaretic acid (NDGA) (data not shown). However, PLPC and α-tocopherol alone had no effect on the adhesion molecule expression. These data imply a requirement for oxidized modification in the PLPC-mediated inhibitory effect. To further examine the role of oxidation, PLPC was oxidized in the presence of 5 μM $CuSO_4$ and a high level of oxidation ascertained by mass spectrometry. When the oxidized PLPC, but not non-oxidized PLPC, was added to cells simultaneously with CRP, E-selectin expression was abrogated (Figure 7B). No period of pre-incubation was required for the effect of oxidized PLPC. In contrast, oxidized PLPC did not inhibit TNF activity (Figure 7B), suggesting a specific effect on CRP. As a control, POPC that had been exposed to $CuSO_4$ did not affect CRP-induced adhesion molecule expression (data not shown) presumably because POPC is less readily oxidized. Taken together, these data strongly indicate that oxidized PLPC is a key molecule accounting for the inhibitory effect of HDL on CRP proinflammatory activity.

Discussion

Previously it has been shown that CRP induces the cell-surface expression of adhesion molecules in HUVEC in the presence of serum, suggesting a proinflammatory action of CRP [4]. We were able to confirm and extend the previous findings and show that E-selectin, VCAM-1 and ICAM-1 are all induced in HUVEC in the absence of serum following stimulation with CRP in HUVEC conditioned medium. Thus, the CRP-induced adhesion molecule expression provides us with a reliable model for investigation of CRP proinflammatory activity *in vitro*. We demonstrate that the proinflammatory activity of CRP can be completely abolished by native HDL at physiological levels. In addition, reconstituted HDL, composed of lipoprotein apoA-I with PLPC as the sole phospholipid, also profoundly inhibited the CRP-induced expression of E-selectin, VCAM-1 and ICAM-

1. Consequently, the physiological significance of these findings was confirmed by the inhibition of CRP induced adherence of U937 cells to aortic EC in the presence of HDL (Figure 5). These findings thus reveal a novel function of HDL to neutralise CRP-mediated proinflammatory activity on vasculature.

One important finding in the present report is that the inhibitory effect of HDL on CRP differed in several aspects from the effect of HDL on cytokine-induced adhesion molecule expression, suggesting at least two mechanisms of protection against endothelial activation and vascular inflammation by HDL. Interestingly, PLPC alone in the form of SUVs had equivalent inhibitory activity to rHDL, whereas neither lipid-free apoA-I nor POPC had any inhibitory effect. These data differ from previous findings that phospholipids alone were unable to mimic the inhibitory effect of whole HDL particles on the cytokine-induced adhesion molecule expression [17], implying a specific role of phospholipids in the protective capacity of HDL against CRP's proinflammatory action.

The finding that pre-conditioning by incubation with HUVEC was required for the inhibitory effect of PLPC or HDL on CRP-induced adhesion molecule expression (Figure 6A), suggested that pre-conditioning converts the HDL or PLPC from an inactive form to a form that has inhibitory activity. To investigate whether endothelial lipases are involved in this process, we used tetrahydrolipstatin, a specific inhibitor of lipases. We found that the anti-inflammatory activity of PLPC was not affected by treatment of cells with the lipase inhibitor (data not shown). Furthermore, the addition of up to 100 μM phosphorylcholine, the product of lipase, to the medium had no effect on the CRP's activity (data not shown). Thus, hydrolysis of PLPC is unlikely to account for the anti-inflammatory activity of PLPC or HDL. A recent report that CRP binds to the phosphorylcholine moiety of oxidized phospholipids but not to unoxidized phospholipids [20] indicated that oxidation may be the mechanism converting PLPC to an active form. In support of this proposition, the anti-oxidants α-tocopherol and NDGA were able to completely abolish the inhibitory effect of PLPC. Additionally, oxidized PLPC inhibited the proinflammatory effect of CRP without requiring preincubation with the cells, suggesting an anti-inflammatory potential of this phospholipid. Although oxidized phospholipids, especially within oxidised-LDL, are generally considered as proinflammatory agonists, recent reports have shown that some oxidized phospholipids indeed inhibit LPS-induced upregulation of inflammatory genes

21;22. We report here that oxidized PLPC was a key molecule in mediating the inhibitory effect of HDL on CRP proinflammatory activity. Oxidation of phospholipids could result in a conformational change that reveals 'cryptic' binding sites to CRP [20]. Thus, the interaction of HDL with EC may expose CRP binding sites by oxidation of the phospholipids within the HDL particles, leading to a competitive inhibition of the interaction between CRP and EC.

The mechanism of interaction between CRP and EC is unknown. FcγRI and FcγRII, the high and low affinity IgG receptors respectively, have been found to bind to CRP, and the latter receptor has been proposed to be the major CRP receptor in phagocytic cells [23]. FcγRII expression is very low on the surface of EC but has been reported to be upregulated on cytokine-stimulated EC [24]. However, the increased expression was time-dependent requiring 2-3 days exposure to cytokine and thus was unlikely to have occurred during the short exposure of HUVEC to either TNF or CRP shown here. Further studies are required to identify whether a specific receptor for CRP exists on EC.

In summary, we have shown a novel function of HDL that, via oxidation of its principal phospholipid, neutralises the proinflammatory potential of CRP in EC, revealing a balance between anti- and pro-inflammatory actions within the vascular wall. Factors that decrease the quantity and/or quality of HDL such as obesity, diabetes, and age are often associated with increased CRP concentration [8,9,25-27], which could ultimately disturb the anti- and pro-inflammatory balance to contribute the development of inflammatory cardiovascular diseases. Therefore, the data presented here has important implications for the development of new strategies to prevent atherosclerosis and associated cardiovascular diseases.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

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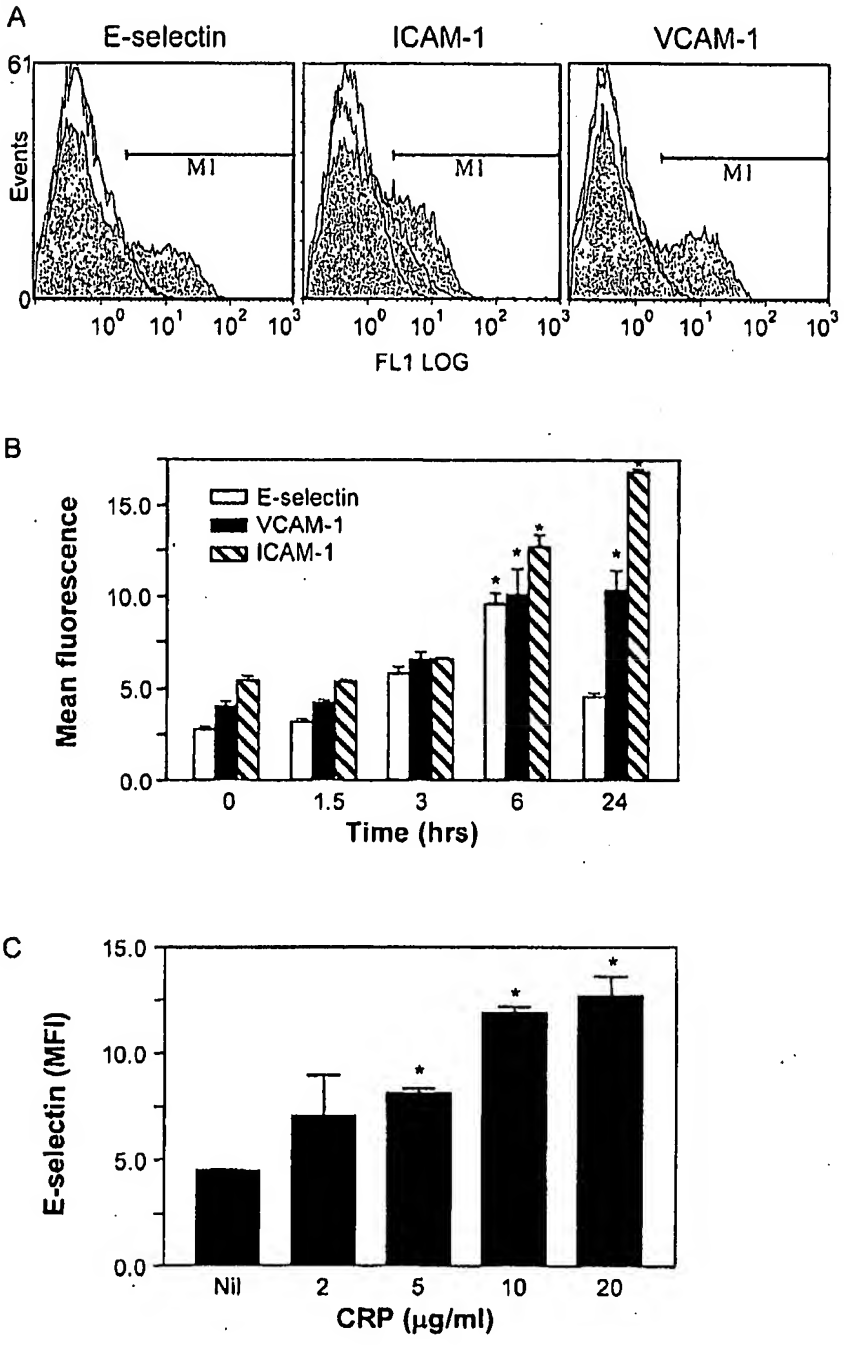
Dated this 21st day of January 2004.

MedVet Science Pty. Ltd.,
By its Patent Attorneys
Davies Collison Cave

Figure 1


A
E-selectin
ICAM-1
VCAM-1
61
Events
0
M1
M1
M1
10^0 10^1 10^2 10^3
FL1 LOG
B
Mean fluorescence
15.0
10.0
5.0
0.0
E-selectin
VCAM-1
ICAM-1
0
1.5
3
6
24
Time (hrs)
C
E-selectin (MFI)
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10.0
5.0
0.0
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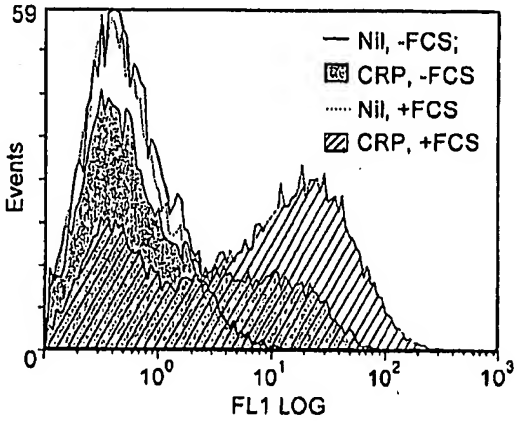
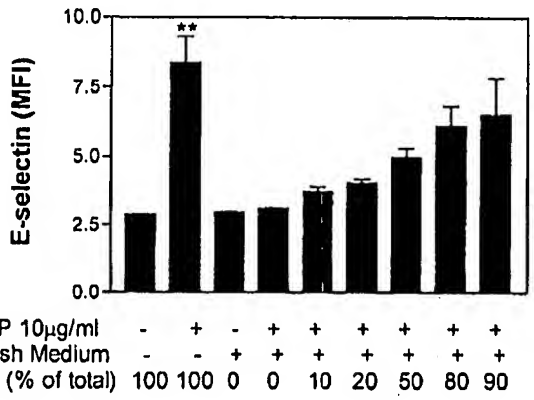
2
5
10
20
CRP (µg/ml)

Figure 2

A


59
Events
0
Nil, -FCS;
CRP, -FCS
Nil, +FCS
CRP, +FCS
10^0
10^1
10^2
10^3
FL1 LOG

B


E-selectin (MFI)
10.0
7.5
5.0
2.5
0.0
**
CRP 10µg/ml - + - + + + + + +
Fresh Medium - - + + + + + + +
CM (% of total) 100 100 0 0 10 20 50 80 90

C

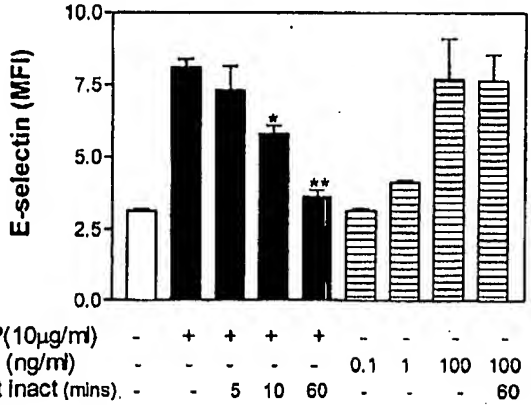
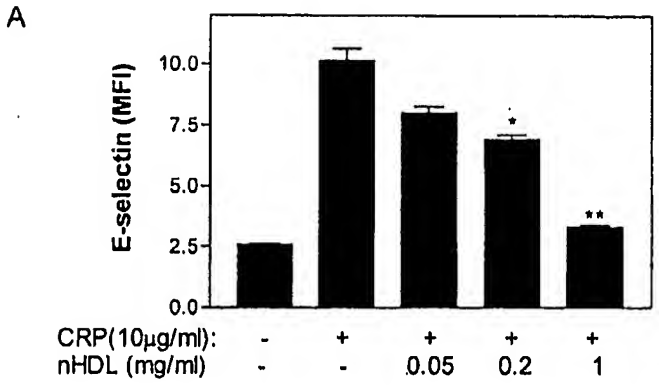
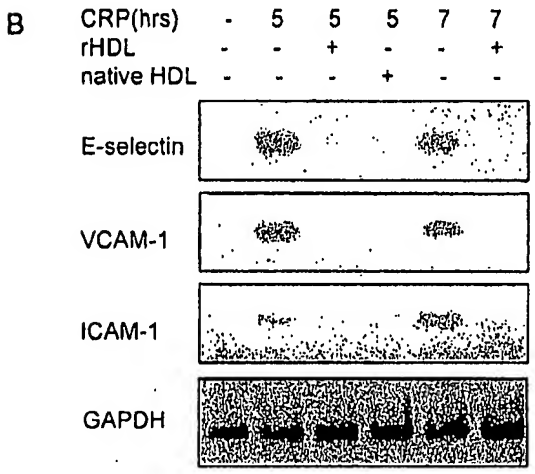

E-selectin (MFI)
10.0
7.5
5.0
2.5
0.0
*
**
CRP(10µg/ml) - + + + + - - - -
LPS (ng/ml) - - - - - 0.1 1 100 100
Heat inact (mins). - - 5 10 60 - - - 60

Figure 3


A
E-selectin (MFI)
10.0
7.5
5.0
2.5
0.0
*
**
CRP(10μg/ml): - + + + +
nHDL (mg/ml) - - 0.05 0.2 1


B
CRP(hrs) - 5 5 5 7 7
rHDL - - + - - +
native HDL - - - + - -
E-selectin
VCAM-1
ICAM-1
GAPDH

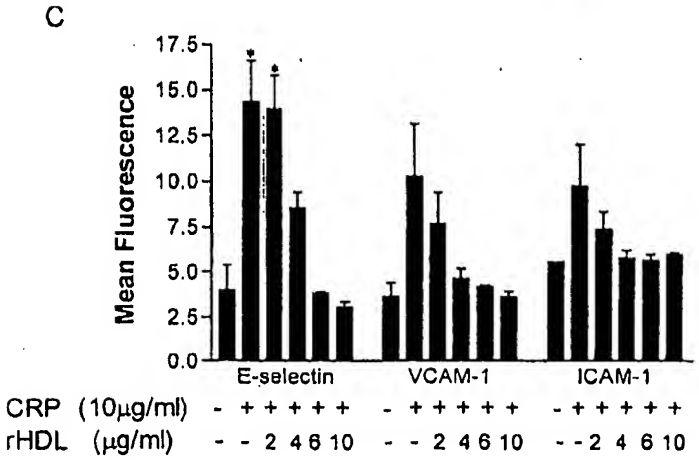
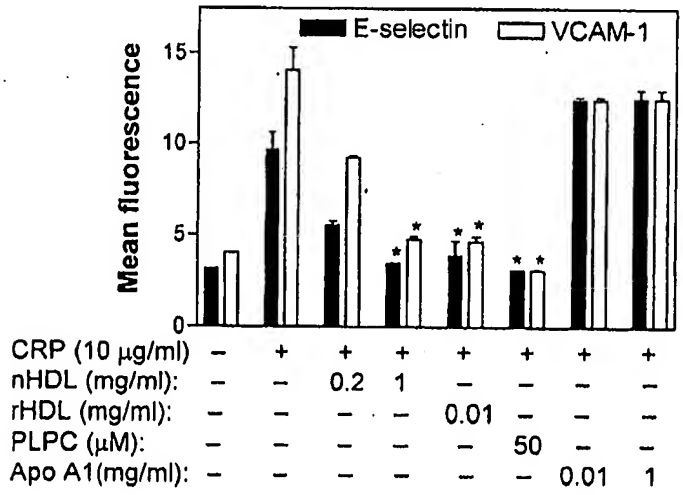
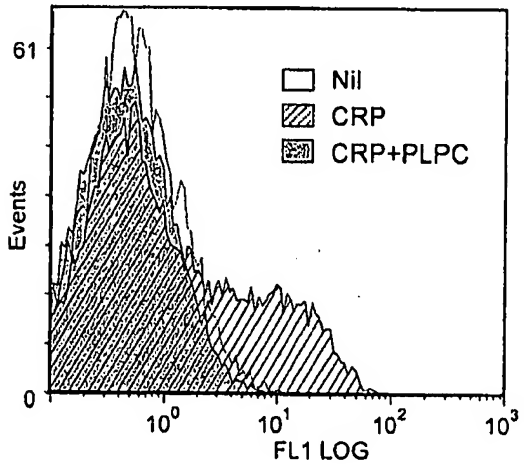

C
Mean Fluorescence
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
*
*
E-selectin
VCAM-1
ICAM-1
CRP (10μg/ml) - + + + + + - + + + + + - + + + + +
rHDL (μg/ml) - - 2 4 6 10 - - 2 4 6 10 - - 2 4 6 10

Figure 4

A


E-selectin
VCAM-1
Mean fluorescence
15
10
5
0
CRP (10 μg/ml) – + + + + + + +
nHDL (mg/ml): – – 0.2 1 – – – –
rHDL (mg/ml): – – – – 0.01 – – –
PLPC (μM): – – – – – 50 – –
Apo A1(mg/ml): – – – – – – 0.01 1

B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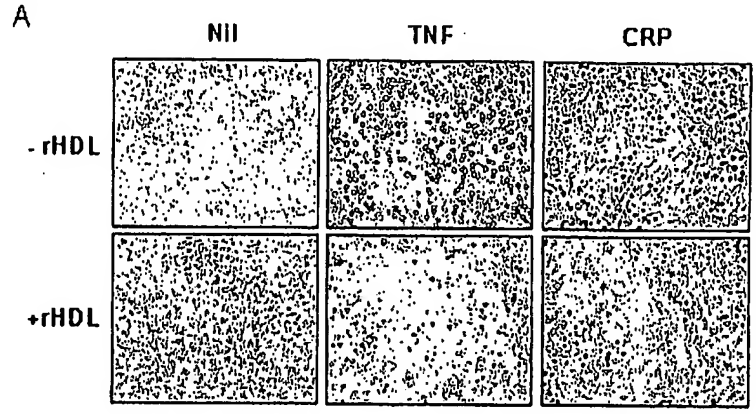
Events
0
Nil
CRP
CRP+PLPC
10^0
10^1
10^2
10^3
FL1 LOG

Figure 5

A


Nil
TNF
CRP
-rHDL
+rHDL

B

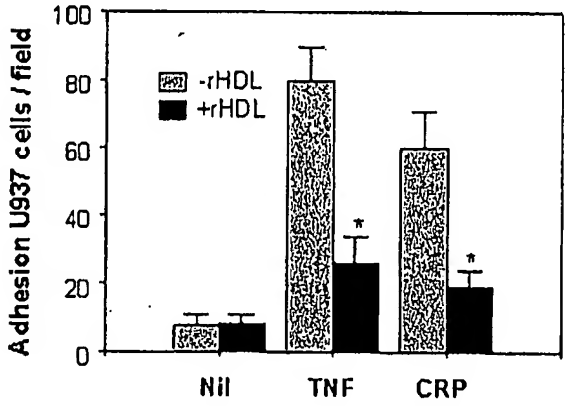
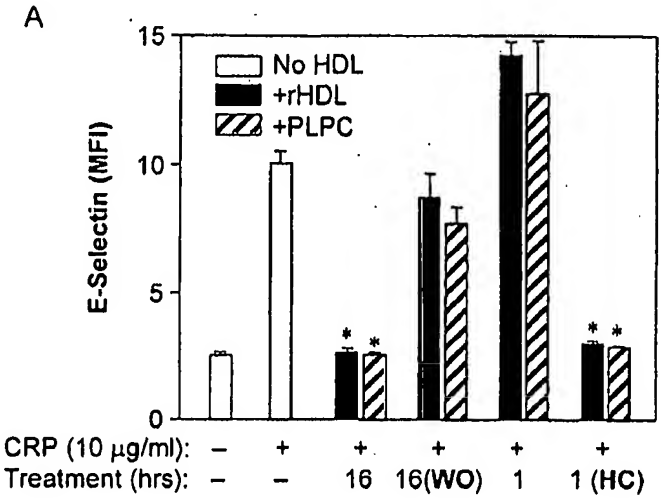

Adhesion U937 cells / field
100
80
60
40
20
0
-rHDL
+rHDL
*
*
Nil
TNF
CRP

Figure 6


A
15
10
5
0
E-Selectin (MFI)
No HDL
+rHDL
+PLPC
CRP (10 μg/ml): – + + + + +
Treatment (hrs): – – 16 16(WO) 1 1 (HC)

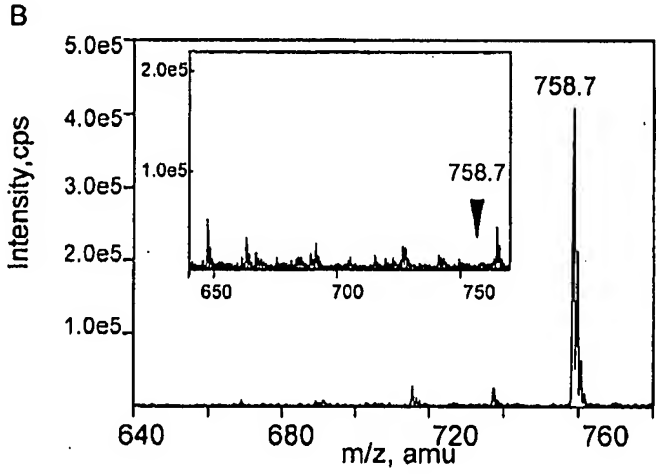
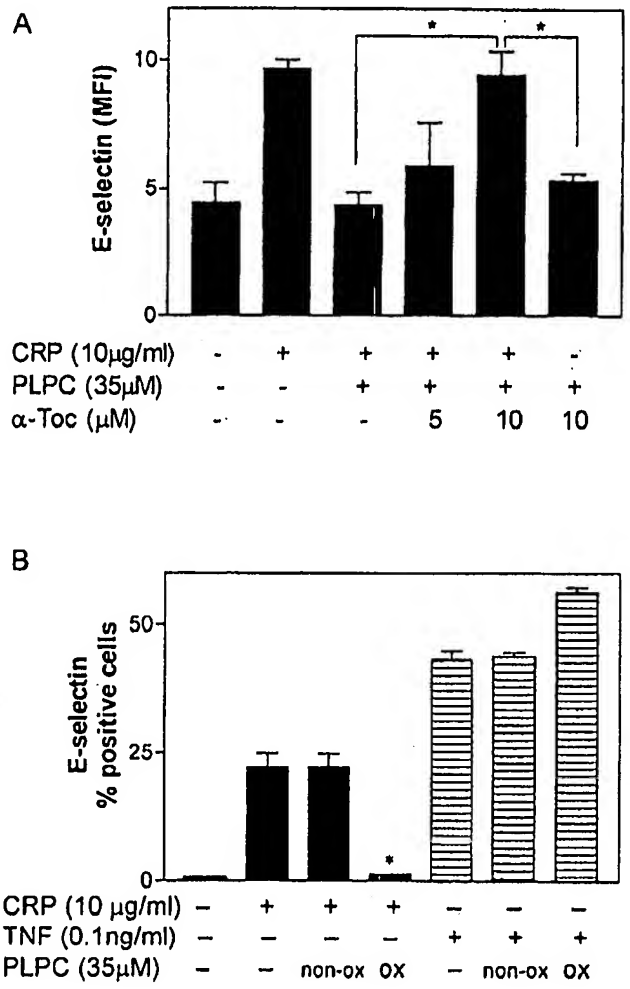

B
5.0e5
4.0e5
3.0e5
2.0e5
1.0e5
Intensity,cps
2.0e5
1.0e5
758.7
758.7
650
700
750
640
680
720
760
m/z, amu

Figure 7


A
E-selectin (MFI)
10
5
0
*
*
CRP (10µg/ml) - + + + + -
PLPC (35µM) - - + + + +
α-Toc (µM) - - - 5 10 10
B
E-selectin
% positive cells
50
25
0
*
CRP (10 µg/ml) – + + + – – –
TNF (0.1ng/ml) – – – – + + +
PLPC (35µM) – – non-ox ox – non-ox ox